A CYTOTOXIN FROM BLEPHARISMA

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When a few paramecia were added to a concentrated suspension of *Blepharisma undulans* in a Cartesian diver, they were injured, began to rotate, and after swelling, died, although the Blepharisma remained normal and active (Giese and Zeuthen, 1949). A few individuals from a Blepharisma culture were placed with a lot of paramecia with no ill effect. An attempt was made to determine what caused the injury to Paramecium placed in a concentrated culture of Blepharisma. The results are described below.

Experimental

Cultures of Blepharisma were grown as previously described (Giese, 1938b). Practically all the other organisms were grown in lettuce infusions of the same type (0.05 per cent lettuce, buffered at pH 7.0 or 8.0), or obtained from wild cultures. *Paramecium multimicronucleatum* for division studies was grown as previously described (Giese, 1945).

In the first experiment the culture of Blepharisma was handled with great care and the animals were gently centrifuged down into the cone of a centrifuge tube. The supernatant was carefully withdrawn and after a dense suspension was available, some paramecia were added. They were in no way adversely affected. It was therefore apparent that when Blepharisma individuals are handled with care they do not liberate any substance injurious to Paramecium. The inference may be drawn that in the pipetting of the suspension of Blepharisma into the diver some individuals may have been injured. To test this possibility individuals in a dense culture of Blepharisma were fragmented by sucking the animals up into a pipette partially blocked by cotton fibers, making a "brei." In this process the animals were torn open and the fluid became pinkish.

Paramecia added to the brei reacted violently by reversed ciliary action and then quickly began moving and died. In a freshly prepared brei, the time from immersion to killing was only a few minutes. A Paramecium-brei similarly prepared was not toxic to Blepharisma nor was a Didinium-brei toxic to Paramecium. Therefore Blepharisma presents a special case worthy of further study.

Questions arise as to the nature and properties of the material liberated by Blepharisma (hereafter called the toxin without any implications other than that it is a poison of organismal origin). It is desirable to know whether the toxin is

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selectively injurious to Paramecium or whether it is generally toxic to organisms. Secondly, the possible function of this toxin is also of interest. Thirdly it is desirable to identify the toxin with some cellular constituent. Experiments attempting to answer these inquiries are described below.

To determine if the toxin liberated by Blepharisma is specific to Paramecium or generally injurious, a wide variety of protozoa were tried. In no case were the protozoans found to be resistant to the Blepharisma-extract, although some were more susceptible than others. *Frontonia leucas* was found to be very susceptible, and it and *Urocentrum turbo* were more susceptible than *Colpidium colpoda* placed in the same brei. The latter was more susceptible than *Paramecium multimicronucleatum* and *P. aurelia. Paramecium bursaria* (green), *Stylonychia curvata, Euglena gracilis, Amoeba proteus* and *Actinosphaerium eichhorni* were found to be more resistant that *P. multimicronucleatum.* Even Rotifers were observed to be affected. Not alone are infusorians injured. Blastulae and gastrulae of the sea urchin, *Stronglyocentrotus purpuratus,* were exposed to small amounts of the substance. They ceased swimming and did not recover; in a few hours they had disintegrated. The material liberated by fragmented Blepharisma individuals seems to be a rather general cellular toxin.

To determine whether the toxin were liberated in limited quantities, a succession of additions of paramecia was made and it was found that whereas the second batch was readily killed, thereafter, the time for killing increased until after many additions there appeared to be no injury. The material appeared to be adsorbed or absorbed by the paramecia and so removed from the solution.

Attempts were made to wash paramecia free of the toxin when they had shown only the first signs of injury, for example, reversed ciliary activity. In no case was the injury reversible, but became more and more pronounced until the paramecia died. Therefore, the toxin seems to become firmly attached.

To determine whether the toxin is injurious to Blepharisma itself, individuals were exposed to a freshly prepared brei. They were not affected and, in fact, they began to clean up the fragments of the corpses as could be seen by the deep red vacuoles within them. Some become giants (see Giese, 1938b, for an account of gigantism in this species). They seem unaffected by having the smaller fragments of their fellows inside them and the toxic material outside. Division was observed to occur and a healthy culture was established. Since many very minute living individuals were also observed in a culture fragmented by passage through cotton fibers, it seems likely that some of the fragments regenerate. The conclusion may be drawn that the material liberated by fragmented Blepharisma while toxic to other forms, is not toxic to itself.

Since the exudate of Blepharisma is so toxic one wonders whether it functions in preventing attack by other organisms. In that case one might expect that carnivorous protozoans would avoid attacking Blepharisma. To test this, carnivores were placed with Blepharisma. *Didinium nasutum,* a particularly voracious ciliate, which attacks Paramecium and Colpidium, avoids Blepharisma. Didinium will starve to death in the midst of a rich culture of Blepharisma but also ignores such colorless forms as Stylonychia. Another ciliate, *Woodruffia metabolica,* also attacks *Paramecium* but ignores Blepharisma as well as many other ciliates. Also the suctorian *Podophrya fixa* feeds upon Paramecium and Colpidium but starves in a culture of Blepharisma. At about the time when it appeared likely that no carnivores would eat Blepharisma, Actinosphaerium was tried. Not only did this heliozoan feed upon Blepharisma but it did so voraciously and individuals of the latter were not only engulfed but digested. Almost as soon as a suspension of Blepharisma was added some were caught in the extended axopodia of the heliozoan. Sometimes on struggling they succeeded in breaking loose, but more often they did not. Within a few minutes they were engulfed in the streaming protoplasm and enclosed in a vacuole which was drawn towards the body. After several hours some individuals of Actinosphaerium had as many as twelve deep red vacuoles. After several more hours they were surrounded with red fecal deposits.

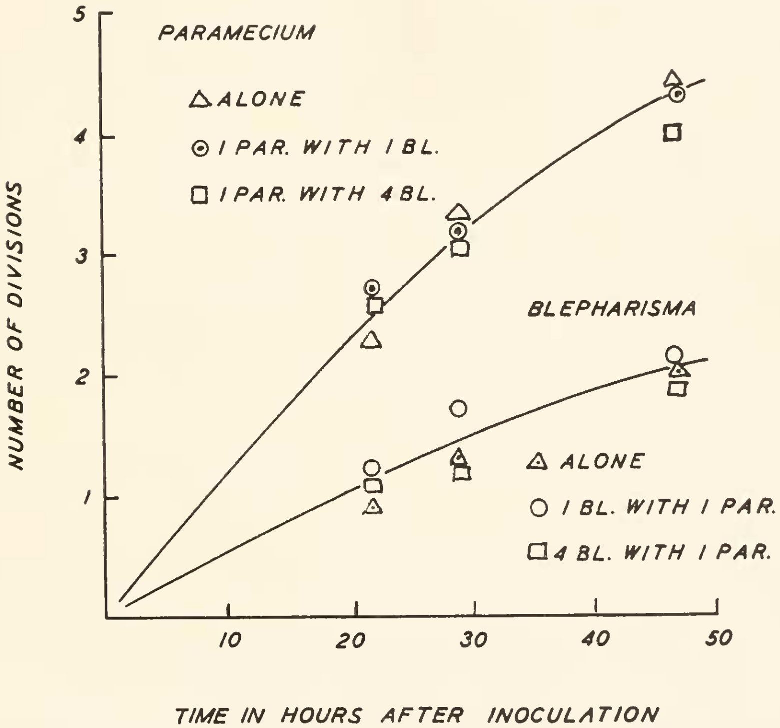

FIGURE 1. Comparison of division-rates of Paramecium in the presence and absence of Blepharisma. Three sets of eight cultures each were used for these determinations. In addition another series in which two instead of four Blepharisma were studied and still another with eight Blepharisma. All the experiments indicated the same result.

If one observes the food vacuoles within an Actinosphaerium, one will see that the contents decrease in size and become more intensely colored. As digestion proceeds the vacuoles do not seem to change hue, always appearing red. Sometimes the fluid within the vacuole turns pink. However the protoplasm of Actinosphaerium never takes on a reddish color. Actinosphaerium fed on Blepharisma continues to grow and divide. Whether division would go on at the same rate as on other food could not be determined since division occurred in such an erratic manner. The latter is probably due to the fact that Actinosphaerium is multinucleate and may grow to a large size before dividing. The experiments with Actinosphaerium demonstrate that in spite of its toxin, Blepharisma is not necessarily protected from carnivores.

Another possible function of the toxin in Blepharisma suggests itself for testing. Perhaps the toxin excludes other species of animals when it accumulates in a culture during growth. This could be tested by growing Blepharisma together with another species in the same culture. Such experiments were performed with Paramecium and Blepharisma. A single specimen of *Paramecium multimicronucleatum* and of Blepharisma placed together in a tube of culture medium grew at about the same rate at controls grown separately. A single specimen of Paramecium placed with four individuals of Blepharisma also grew as well as the control in the absence of Blepharisma. The data are summarized in Figure 1. The conclusion may be drawn that if something is exuded from Blepharisma during growth it is insufficient to prevent paramecia from growing at least as rapidly as they would in the absence of Blepharisma.[1]

The possibility that the toxin might be the reddish pigment suggests itself since in experiments on the effects of the brei on paramecia and some of the other colorless forms it was noted that the animals became reddish after they were injured. If this were true then if the pigment were first destroyed by bleaching one might expect the brei of such animals to be innocuous. Accordingly two experiments were tried. In the first the individuals in a culture of Blepharisma were killed and disrupted by exposure to visible light (for method see Giese, 1946) and the light treatment was continued until relatively little color remained. To this material, paramecia were added and it was found that there was little if any observable effect on them. In the second set of experiments the culture of Blepharisma was first bleached by exposing it to weak light (Giese, 1938a). This was accomplished by placing it near a 6-watt daylight fluorescent lamp cooled by a fan. From the animals bleached for 24 hours a brei was made and it proved completely non-toxic to Paramecium. The material which is toxic is therefore photolabile. However the pigment might merely act as a photosensitizer to some other constituent such as a protein or fat of the cell which when affected becomes toxic. This might be answered by separating the pigment from the fats and proteins of the cell.

The pigment was next extracted with absolute alcohol (Emerson, 1935) from animals concentrated into a small red mass by centrifuging. It was then freed from particulate detritus by centrifuging and dried in a water bath and was reextracted with absolute alcohol and again dried in another dish. It was then extracted with water. Only a portion of the original pigment went into aqueous solution which was clear and reddish. From the solubility properties it would appear that the toxic substance of Blepharisma is not related to the killer substance paramecin (Sonneborn, 1948; van Wagtendonk, 1948) produced by some strains of *Paramecium aurelia*.

[1] One unexpected result of growing Paramecium and Blepharisma together is the formation of Blepharisma giants which eat Paramecium. This occurs only in cultures with *P. aurelia;* at least it was never observed in the cultures with *P. multimicronucleatum*. It is probable that the latter species is just too large to be engulfed since even when specimens of Blepharisma had become very much enlarged as a result of feeding on smaller species, they did not succeed in ingesting the larger species of Paramecium.

Specimens of Paramecium introduced into diluted aqueous pigment solution reacted much as they did to the crude material from crushed Blepharisma. They showed very strong reversed ciliary activity, then began to rotate; and as the contractile vacuoles ceased working, the animals became enlarged and died. Upon dying they became distinctly stained with a reddish tinge. While it is not certain that something toxic is not combined with the pigment, such preliminary trials as have been made using absorption column analysis indicate a single substance. The tentative hypothesis is put forth that the pigment is the toxic material. This can only be tested further by purification and study of the pigment. Such experiments are under way.

Summary

1. A brei of fragmented Blepharisma contains some substance which is quite toxic to Paramecium and a variety of other protozoans and to sea urchin larvae, but it is not toxic to Blepharisma itself.

2. Paramecia suspended in a dense culture of Blepharisma are unaffected by the mere presence of Blepharisma.

3. Blepharisma is eaten by Actinosphaerium; therefore the toxin does not protect it from attack and use as food, but it is not eaten by Woodruffia, Podophrya or Didinium.

4. In the presence of Blepharisma, paramecia grow at the same rate as they do alone, indicating that no toxin is secreted during growth.

5. Brei of Blepharisma bleached by light is not toxic to paramecia.

6. The pigment of Blepharisma extracted in alcohol and after drying re-extracted in alcohol and, after another drying, re-extracted in water is highly toxic to paramecia.

7. The tentative conclusion is drawn that the toxin is the pigment or something very closely associated with it.

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